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Hemolysis induced by *Bacillus cereus* sphingomyelinase

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ABSTRACT

Bacillus cereus sphingomyelinase (*Bc*-SMase) induces hemolysis of sheep erythrocytes which contain large amounts of sphingomyelin. We investigated the mechanism of this hemolysis in comparison to that induced by *Clostridium perfringens* alpha-toxin. Pertussis toxin, a Gi-specific inhibitor, *N*-oleoylethernolamine, a ceramidase inhibitor, and dihydrosphingosine, a sphingosine kinase inhibitor, did not inhibit the hemolysis by *Bc*-SMase, but did inhibit that by alpha-toxin. *Bc*-SMase broadly bound to whole membranes, and alpha-toxin specifically bound to the detergent-resistant membrane fractions, lipid rafts. The level of ceramide production induced by *Bc*-SMase in sheep erythrocytes was 6- to 15-fold that induced by alpha-toxin, when the extent of the hemolysis by *Bc*-SMase was the same as that by the toxin. However, the level of ceramide production induced by *Bc*-SMase in SM-liposomes was equal to that triggered by the toxin, when the carboxyl fluorescein-release from liposomes induced by *Bc*-SMase was the same as that induced by alpha-toxin. Confocal laser microscopy showed that treatment of the cells with *Bc*-SMase resulted in the formation of ceramide-rich domains. A photobleaching analysis suggested that treatment of the cells with *Bc*-SMase leads to a reduction in membrane fluidity. These results show that *Bc*-SMase-induced hemolysis of sheep erythrocytes is related to the formation of interface between ceramide-rich domains and ceramide-poor domains through production of ceramide from SM.

1. Introduction

Sphingomyelinase (SMase) is an enzyme which hydrolyses sphingomyelin (SM) to ceramide and phosphocholine. Several types of enzyme with SMase activity have been identified in eukaryotes and prokaryotes. Eukaryotic SMases have been classified according to their pH optima, as acid [1], alkaline [2], and neutral SMases (nSMases) [3,4]. Several pathogenic bacteria, such as *Staphylococcus aureus* [5], *Bacillus cereus* [6], *Leptospira interrogans* [7], and *Listeria ivanovii* [8], produce SMases. These bacterial SMases share sequence homology with eukaryotic nSMases. These observations suggest that bacterial and eukaryotic nSMases share a similar catalytic mechanism and overall structure [9,10].

SMase from *Bacillus cereus* (*Bc*-SMase) with a molecular mass of 34 kDa exhibits enzymatic and hemolytic activities [11]. The enzyme binds specifically to membranes of sheep erythrocytes and selectively hydrolyses SM in membranes of the erythrocytes. It has been reported that Ca^{2+} and Mn^{2+} enhanced binding of the enzyme to the erythrocyte membranes, whereas Mg^{2+} accelerated hemolytic activity together with the breakdown of SM [3,12–14]. We reported that divalent metal ions were effective in stimulating the activity of *Bc*-SMase in the order $Co^{2+} \geq Mn^{2+} \geq Mg^{2+} \gg Ca^{2+} \geq Sr^{2+}$, and resolved the structure of *Bc*-SMase bound to Co^{2+}, Mg^{2+} or Ca^{2+} [15]. A crystal analysis showed that *Bc*-SMase has two metal ion-binding sites in a long horizontal cleft across the molecule; the central region of the cleft is the binding-site of one Mg^{2+} or two Co^{2+} and the side of the cleft is the binding-site of one divalent metal ion [15]. We also showed that the metal ions in the central region play an important role in the enzymatic activity of *Bc*-SMase. However, the role of the metal ions at the side remains unclear. We also provided evidence that the exposed loop from Asn-92 to Pro-98 participates in the binding to the substrate and perhaps membranes [15].

Little is known about how the mechanism of hemolysis of sheep erythrocytes is induced by *Bc*-SMase. We have reported that the alpha-toxin-induced hemolysis of rabbit erythrocytes is due to activation of the metabolism of glycerophospholipids through a GTP-binding protein [16,17], and that the toxin triggers hemolysis though the activation of an endogenous SMase via a GTP-binding protein in sheep erythrocytes [18]. Furthermore, we reported that alpha-toxin-induced activation of the metabolism of C24:1-SM through Gi in detergent-resistant membrane fractions (DRM) of sheep erythrocytes, especially the formation of S1P, is closely involved in hemolysis [19]. Therefore, to elucidate the mechanism of hemolysis by *Bc*-SMase, we compared it with that induced by alpha-toxin.

Abbreviations: B. cereus, *Bacillus cereus*; *Bc*-SMase, *Bacillus cereus* sphingomyelinase; *C. perfringens*, *Clostridium perfringens*; DRM, detergent-resistant membranes; DSF, detergent-soluble fractions; SM, sphingomyelin; S1P, sphingosine 1-phosphate; MβCD, methyl-β-cyclodextrin; TBS, tris-buffered saline; PT, pertussis toxin; DHS, dihydrosphingosine; CF, carboxyl fluorescein; FRAP, fluorescence recovery after photobleaching

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2. Materials and methods

2.1. Drugs

Sheep erythrocytes were purchased from Nippon Bio-Test Laboratories, Japan. *N*-oleoylethanolamine (*N*-OE), cardiolipin, methyl-β-cyclodextrin (MβCD) and 5(6)-carboxyfluorescein diacetate (CF) were obtained from Sigma-Aldrich Chemical, USA. Dihydrosphingosine (DHS) was from Calbiochem-Novabiochem, USA. Leupeptin and pepstatin were obtained from Chemicon International, USA. 1-O-*n*-Octyl-β-D-glucopyranoside was purchased from Nacalai Tesque, Japan. [γ-^{32}P]ATP (4,500 Ci/mmol) was supplied by ICN Biochemicals, USA. All other drugs were of analytical grade.

2.2. Preparation of B. cereus sphingomyelinase and the variant enzyme (E53A)

The expression and purification of recombinant *Bc*-SMase and E53A were performed as described previously [15].

2.3. Site-directed mutagenesis

The transformer site-directed mutagenesis kit (BD Biosciences, USA) was used with the primer E53A: GTTATTTTAAATGCCGTGTTTGATAATAGC, to prepare the modified plasmid. The genetic sequence of *Bc*-SMase in each plasmid was confirmed with an ABI310 PRISM™ genetic analyzer (Applied Biosystems, USA).

2.4. Preparation of C. perfringens alpha-toxin and the variant enzyme (H148G)

The expression and purification of recombinant alpha-toxin and H148G were performed as described previously [18].

2.5. Preparation of sheep erythrocytes

Sheep erythrocytes were suspended in 0.02 M Tris–HCl buffer (pH 7.5) containing 0.9% NaCl (TBS), and centrifuged at 1100 ×*g* for 3 min. The erythrocytes were washed by the centrifugation three times. The number of erythrocytes was determined with a cell counter (Celltac; Nihon Kohden, Japan).

2.6. Determination of hemolytic activity

Bc-SMase or alpha-toxin was incubated with sheep erythrocytes (12×10^{10} cells/ml) in TBS at 37 °C for 30 min, and the cells were chilled at 4 °C. The hemolysis of the erythrocytes was measured, as described previously [18]. Hemolysis was expressed as a percentage of the amount of hemoglobin released from 0.1 ml of erythrocytes suspended in 0.4 ml of 0.4% NaCl.

2.7. Determination of ceramide

Levels of ceramide in the erythrocytes treated with *Bc*-SMase or alpha-toxin were measured as described previously [18].

2.8. Sucrose gradient fractionation

Separation of DRM was carried out by flotation-centrifugation with a sucrose gradient. Sheep erythrocytes were incubated with 40 μM of E53A (*Bc*-SMase mutant) or H148G (alpha-toxin mutant) at 37 °C for various periods in TBS containing 0.25% (w/v) gelatin (GTBS). The cells were washed with TBS and treated with 0.5% Triton X-100 for 30 min at 4 °C in TBS containing the protease inhibitor cocktail (Nacalai Tesque) and sonicated for 30-s pulses using a tip-type sonicator. The lysates were adjusted to 40% sucrose (w/v), overlaid with 2.4 ml of 36% sucrose and 1.2 ml of 5% sucrose in TBS, centrifuged at 45,000 rpm (250,000 ×*g*) at 4 °C for 18 h in a SW55 rotor (Beckman Instruments, USA), and fractionated from the top (0.4 ml each, a total of 10 fractions). The aliquots were subjected to SDS-PAGE and Western blotting using each specific antibody.

2.9. Immunoblot analysis of DRM marker proteins

Aliquots of the flotation sucrose gradient fractions were heated in 2% SDS-sample buffer at 100 °C for 3 min. The samples were electrophoresed on an SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with TBS containing 2% Tween 20 and 5% skim milk and incubated with a primary antibody such as anti-alpha-toxin (1:1000), anti-*Bc*-SMase (1:1000), or anti-flotilin-1 (1:1000) in TBS containing 1% skim milk, then with a horseradish peroxidase-conjugated secondary antibody, and finally with an enhanced chemiluminescence analysis kit (GE health science, USA). The level of cholesterol was assayed spectrophotometrically using a diagnostic kit (Cholesterol C-test; Wako, Japan).

2.10. Preparation of liposomes

SM (Nacalai Tesque) from bovine brain and cholesterol (1:1) in chloroform-methanol (2:1 v/v) were dried with N_2 gas, resuspended in TBS containing 0.1 M CF. The liposome suspensions were centrifuged at 22,000 ×*g* for 15 min at 4 °C to remove the nonencapsulated marker, and washed three times by centrifugation. The resulting liposomes were suspended in 200 μl of TBS.

2.11. The SM-liposome-disruption activity

The SM-liposome-disruption activity was evaluated at the amount of released-CF in the test aliquot. The SM-liposomes in TBS containing 1 mM $MgCl_2$ were incubated with *Bc*-SMase or alpha-toxin for 30 min at 37 °C. 100% CF release was defined as the fluorescence intensity that was gained upon exposure of liposomes to 2% Triton X-100 at 37 °C for 30 min.

2.12. Fluorescence microscopy

A confocal fluorescence microscope (A1; Nikon, Japan) was used. The excitation wavelength was 488 nm for BODIPY FL C_{12}-SM (Molecular probes, USA) (BODIPY-SM). The fluorescence signals were simultaneously collected using NIS-Elements C (Nikon software) into a channel using bandpass filters of 525/50. The objective used in all experiments was a 60× oil immersion, CFI Plan Apo VC 60× oil/1.40 objective (Nikon). The objective lens was used with a zoom factor of 2. The experiments were performed at room temperature.

2.13. Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP), a technology used to measure the lateral mobility of membranes, was performed with a Nikon A1R confocal laser scanning microscope, according to the manual. Sheep erythrocytes stained with 2 μM BODIPY-SM were plated on 35-mm glass-bottomed dishes (MatTek, USA). The treated cells were spread on 0.3% agar containing TBS. The photobleaching was performed in a 1.5 μm, visually uniform region of the cell membranes. Bleaching was performed with 5% laser intensity for a duration of approximately 1 s (5 scans of the laser) to achieve 50% bleaching of the BODIPY fluorescence. After photobleaching, images were acquired 200 times at 1-s intervals.

2.14. Determination of BODIPY-SM and -ceramide

Sheep erythrocytes (15×10^{10} cells/ml) were stained with 2 μM BODIPY-SM. The cells were incubated with *Bc*-SMase in GTBS at 37 °C for 30 min, and the reaction was stopped by chloroform-methanol (2:1). The lipids were extracted by the method as reported previously [19]. The extracted lipids were developed by normal-phase TLC with chloroform-methanol-DW (65:25:4, v/v). Labeled lipids on the plate were visualized with a Bio-Imaging Analyzer FLA-2000 using the following filter: excitation 473 nm and emission 580 nm (Fujifilm Co., Tokyo, Japan). The mobility of BODIPY-labeled SM and ceramide on TLC corresponded to that of SM from bovine brain and $C_{24:1}$-ceramide (Avanti Polar Lipid, USA).

2.15. Immunofluorescence staining and confocal imaging

Sheep erythrocytes (15×10^{10} cells/ml) were stained with 2 μM BODIPY-SM. The cells were incubated with *Bc*-SMase in GTBS at 37 °C for 30 min, and the reaction was stopped by 1.0% paraformaldehyde at room temperature. For antibody labeling, the cells were incubated at room temperature for 10 min in 50 mM NH_4Cl in phosphate buffered saline (PBS). After being washed with PBS, the cells were incubated at room temperature for 60 min with PBS containing 4% BSA, followed by mouse anti-ceramide antibody in PBS for 60 min. After a wash with PBS, the cells were incubated at room temperature for 60 min with Alexa Fluor 546-conjugated anti-mouse IgG. The treated cells were spread on 0.3% agar containing TBS.

2.16. Statistical analysis

All values are expressed as means ± SEM. The significance differences between experimental and control groups were determined with Student's *t*-test. $P<0.01$ was considered statistically significant.

3. Results

3.1. Effect of pertussis toxin on hemolysis by Bc-SMase and alpha-toxin

We reported that the hemolysis of sheep erythrocytes by alpha-toxin was dependent on the activation of endogenous SMase via Gi [18]. PT is a specific inhibitor of signal transduction via Gi [20–22]. *Bc*-SMase is known to lyse sheep erythrocytes similar to alpha-toxin [11,13]. To compare the effect of PT on the hemolysis by *Bc*-SMase with that by the toxin, erythrocytes preincubated with various concentrations of PT were incubated with *Bc*-SMase or alpha-toxin at 37 °C for 30 min. As shown in Fig. 1A, PT at concentrations of 5 to 20 μg did not significantly inhibit the hemolysis by *Bc*-SMase, but did impede that induced by alpha-toxin in a dose-dependent manner. In addition, *Bc*-SMase hydrolyzed SM in membranes in the presence of 20 μg of PT, but alpha-toxin mostly did not (data not shown).

3.2. Effect of N-OE and DHS on hemolysis induced by Bc-SMase and alpha-toxin

We tested whether hemolysis induced by *Bc*-SMase is dependent on activation of the SM metabolism system. Sheep erythrocytes were preincubated with various concentrations of *N*-OE [23], an inhibitor of ceramidase, or DHS [24], an inhibitor of sphingosine kinase, at 37 °C for 30 min, and incubated with 0.8 μM of *Bc*-SMase or alpha-toxin at 37 °C for 30 min. Treatment of sheep erythrocytes with 100 μM *N*-OE or 20 μM DHS had little effect on the hemolysis by *Bc*-SMase. However, *N*-OE (12.5 to 75 μM) and DHS (5 to 20 μM) inhibited the hemolysis by alpha-toxin in a dose-dependent manner (Fig. 1B and C).

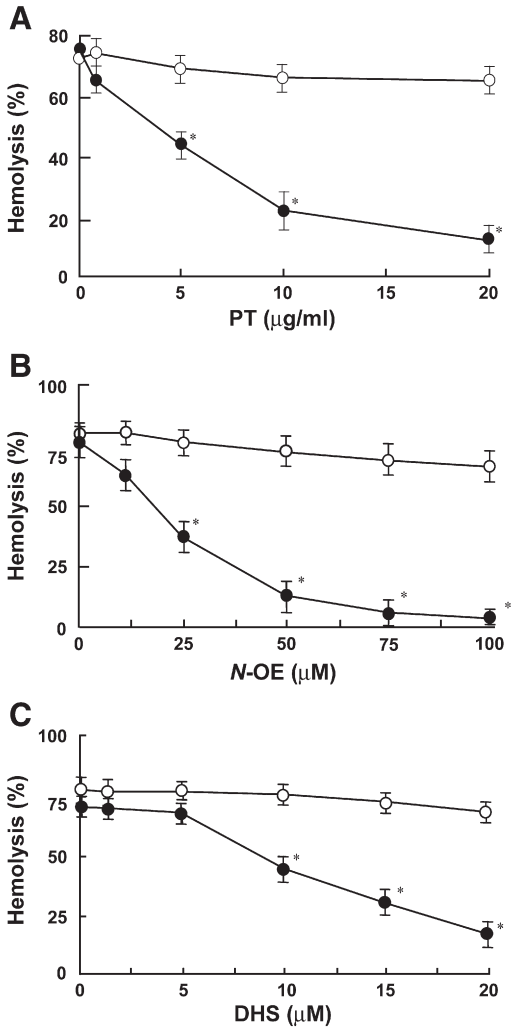

Fig. 1. Effect of several inhibitors on the hemolysis of sheep erythrocytes by *Bc*-SMase or alpha-toxin. Sheep erythrocytes (15×10^{10} cells/ml) were preincubated with various concentrations of PT (A), *N*-OE (B), or DHS (C) at 37 °C for 120, 60, and 30 min, respectively, then incubated with 0.8 μM of alpha-toxin (●) or *Bc*-SMase (○) in TBS containing 0.3 mM $CaCl_2$ at 37 °C for 30 min, and chilled at 4 °C for 10 min. Values are means ± standard deviations for six independent experiments. *$P<0.01$ compared with hemolysis induced by *Bc*-SMase or alpha-toxin alone.

3.3. Binding of Bc-SMase to membranes of sheep erythrocytes

It has been reported that bacterial toxins such as *C. perfringens* beta toxin [25], perfringolysin O [26] and shiga toxin [27] specifically bind to DRM which are implicated in signal transduction. Therefore, to analyze the binding of *Bc*-SMase and alpha-toxin to the erythrocyte membranes, the cells were incubated with E53A (a *Bc*-SMase mutant) and H148G (an alpha-toxin mutant), which bind to erythrocytes, but do not contain the hemolytic and enzymatic activities of PLC and/or SMase [28,29], at 37 °C for 15 min, and treated with 1% TritonX-100 at 4 °C. The Triton X-100-insoluble components were fractionated by sucrose density gradient centrifugation. The fractions were subjected to SDS-PAGE and Western blotting. When the DRM marker in the fractions obtained by the centrifugation was analyzed using anti-flotillin-1, flotillin-1 was detected in the low density fractions (fractions 3–5) and the fractions with a much higher proportion of cholesterol (Fig. 2A). As shown in Fig. 2A, E53A was detected in whole membranes, from DRM (fractions 3–5) to detergent-soluble fractions (DSF, fractions 6–10), and H148G was detected in DRM. The result suggests that *Bc*-SMase broadly binds to sheep erythrocyte membranes, and the toxin binds to the lipid rafts.

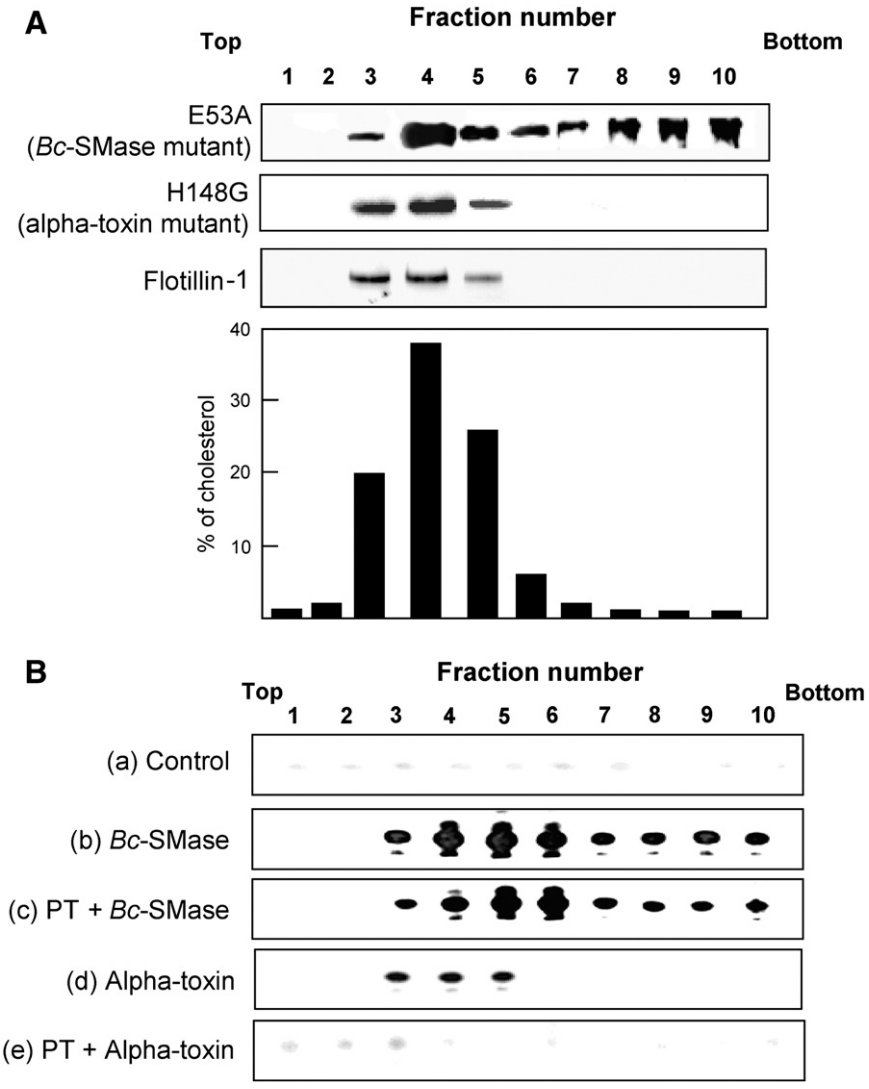

Fig. 2. The binding of *Bc*-SMase or alpha-toxin to membrane of sheep erythrocytes. (A) Sheep erythrocytes (15×10^{10} cells/ml) were incubated with E53A (40 μM) or H148G (40 μM) in GTBS at 37 °C for 30 min and fractionated by sucrose gradient ultracentrifugation. Fractions were collected from the top and subjected to SDS-PAGE, followed by Western blotting using specific antibodies. The amount of cholesterol in the fractions was determined as described in Materials and methods. (B) Sheep erythrocytes (15×10^{10} cells/ml) were pretreated with (c, e) or without (a, b, d) 10 μg/ml of PT at 37 °C for 120 min. The treated erythrocytes were incubated with *Bc*-SMase (b, c) or alpha-toxin (d, e) at 37 °C for 30 min. The Triton X-100-insoluble fractions were separated by sucrose density ultracentrifugation. Ceramide in the fractions was separated by TLC. A typical result from one of five experiments is shown.

We examined whether or not *Bc*-SMase or alpha-toxin triggers the production of ceramide from SM in DRM. We reported that ceramide produced on treatment with the toxin was readily hydrolyzed to sphingosine by ceramidase [18]. Therefore, sheep erythrocytes pretreated with *N*-OE were incubated with *Bc*-SMase or alpha-toxin at 37 °C for 30 min. As shown in Fig. 2B, ceramide in the cells treated with *Bc*-SMase was detected in not only DRM, but also DSF. The formation of ceramide induced by *Bc*-SMase was not inhibited by PT. On the other hand, the ceramide produced in the cells treated with the toxin was mainly detected in DRM. PT inhibited the alpha-toxin-induced formation of ceramide in DRM (Fig. 2B).

3.4. Effect of MβCD on Bc-SMase- or alpha-toxin-induced hemolysis

It has been reported that MβCD selectivity encapsulates cholesterol in cell membranes, but does not deplete other lipids at concentrations below 10 mM [30]. The effect of MβCD on *Bc*-SMase- or alpha-toxin-induced hemolysis of the erythrocytes was investigated. The erythrocytes were incubated with various concentrations of MβCD (2.5–10 mM) at 37 °C for 60 min. The treatment with MβCD resulted in little inhibition of the hemolysis by *Bc*-SMase, but inhibited the hemolysis by alpha-toxin in a dose-dependent manner (Fig. 3).

3.5. Comparison of hemolysis by Bc-SMase with that by alpha-toxin of sheep erythrocytes

We analyzed the relationship between the formation of ceramide and hemolysis by *Bc*-SMase and alpha-toxin. The level of hemolysis by *Bc*-SMase was the same as that by the toxin at concentrations of 0.05–0.8 μM, as shown in Fig. 4A and B. The amount of ceramide produced in response to *Bc*-SMase was about 6- to 15-fold that produced on exposure to alpha-toxin under the conditions. Therefore, it appears that the hemolysis by the former is dependent on the production of ceramide induced by the enzyme, but that by the latter is not.

3.6. Comparison of the disruption of SM-liposomes by Bc-SMase with that by alpha-toxin

To investigate the actions of *Bc*-SMase and the toxin on artificial membranes, the amount of CF released from SM-liposomes treated

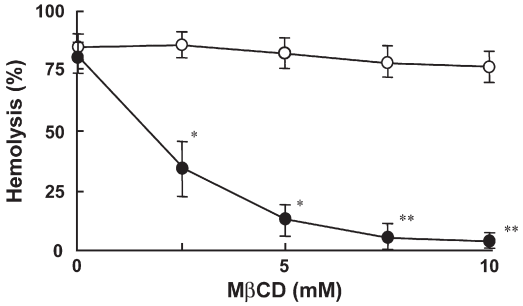

Fig. 3. Effect of MβCD on *Bc*-SMase or alpha-toxin-induced hemolysis. Sheep erythrocytes (15×10^{10} cells/ml) were preincubated with various concentrations of MβCD at 37 °C for 60 min and incubated with 0.8 μM *Bc*-SMase (○) or alpha-toxin (●) at 37 °C for 30 min, then by chilled at 4 °C for 10 min. Hemolysis (%) was evaluated as described in the Materials and methods. Values are means ± standard deviations for three independent experiments. $^{*}P<0.01$ or $^{**}P<0.005$ compared with hemolysis induced by *Bc*-SMase or alpha-toxin alone.

with *Bc*-SMase was compared with that released by alpha-toxin. As shown in Fig. 5, *Bc*-SMase and the toxin at concentrations ranging from 0.5 to 3.0 μM and 3.0 to 30 μM, respectively, had similar effect on CF-release from SM-liposomes and the formation of ceramide in a dose-dependent manner (Fig. 5), indicating that the ability of *Bc*-SMase to hydrolyze SM in membranes was about 10-fold greater than that of alpha-toxin. Thus, it is likely that the disruption of artificial membranes by *Bc*-SMase and the toxin is dependent on the level of production of ceramide from SM in membranes.

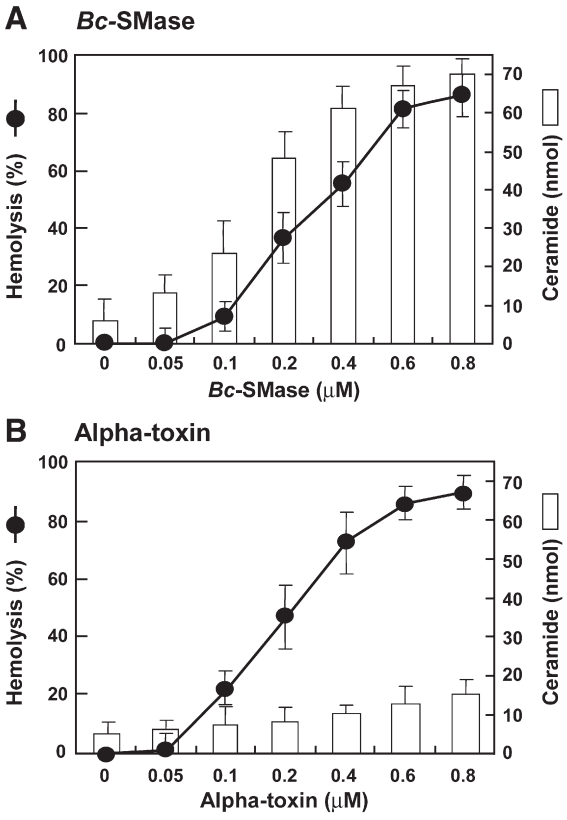

Fig. 4. Comparison of hemolysis and formation of ceramide induced by *Bc*-SMase or alpha-toxin. Sheep erythrocytes (15×10^{10} cells/ml) were incubated with *Bc*-SMase (A) or alpha-toxin (B) at 37 °C for 30 min, and chilled at 4 °C for 10 min. Hemolysis and the formation of ceramide were evaluated as described in Materials and methods. Values are means ± standard deviations for five independent experiments.

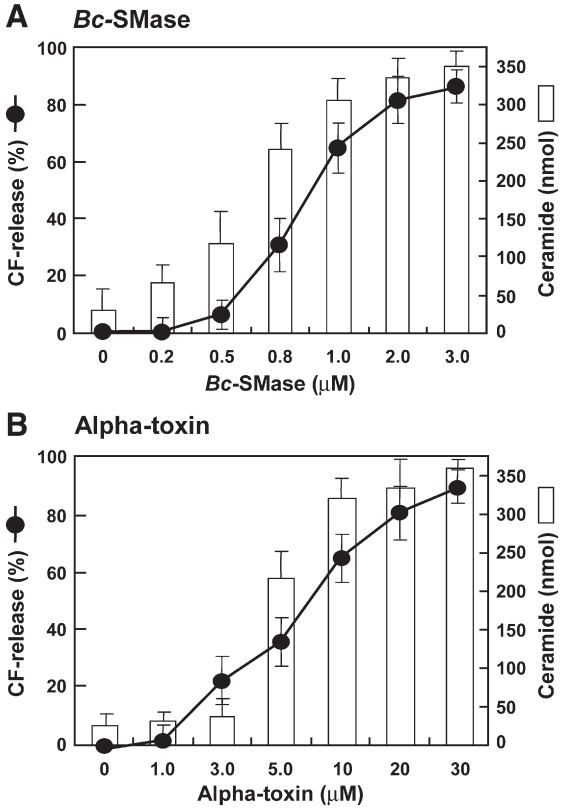

Fig. 5. Comparison of disruption of SM-liposomes and formation of ceramide induced by *Bc*-SMase or alpha-toxin. SM-liposomes were incubated with *Bc*-SMase (A) or alpha-toxin (B) at 37 °C for 30 min. The disruption of SM-liposomes and formation of ceramide were evaluated as described in the Materials and methods. Values are means ± standard deviations for seven independent experiments.

3.7. Localization of ceramide in erythrocyte membranes treated with Bc-SMase

Using confocal microscopy, Montes et al. found that phospholipase C from *Pseudomonas aeruginosa* caused the formation of ceramide-rich domains [31]. To investigate whether *Bc*-SMase induces the accumulation of BODIPY-ceramide formed from BODIPY-SM in the membranes, the erythrocytes stained with BODIPY-SM were incubated with *Bc*-SMase or alpha-toxin at 37 °C. Fig. 6A shows sheep erythrocytes stained with BODIPY-SM. When the erythrocytes were treated with *Bc*-SMase, the domains, in which the fluorescent substances were concentrated, were observed (Fig. 6B, white arrows). However, fluorescent substance-rich domains were not observed in membranes of the cells treated with 0.8 μM alpha-toxin (Fig. 6C). As shown in Fig. 6D, incubation of the BODIPY-SM-stained erythrocytes with 0.8 μM *Bc*-SMase resulted in disappearance of BODIPY-SM and appearance of BODIPY-ceramide in membranes, indicating that BODIPY-SM in the erythrocytes is almost hydrolyzed to BODIPY-ceramide by *Bc*-SMase. Furthermore, treatment of sheep erythrocytes with 0.8 μM *Bc*-SMase at 37 °C for 30 min resulted in the hydrolysis of more than 80% of SM to ceramide in the membranes (data not shown). We investigated whether the concentrated domain of fluorescent substances is corresponding to ceramide-rich domain. BODIPY-SM-stained erythrocytes were incubated with 0.8 μM *Bc*-SMase, and fixed with 1% paraformaldehyde. The domains of fluorescent substances were recognized by anti-ceramide antibody, whereas erythrocytes stained with BODIPY-SM were not reacted with anti-ceramide antibody (Fig. 6E). Treatment of the *Bc*-SMase-pretreated cells with 1.0% paraformaldehyde caused morphological change (shrinkage), but that of untreated cells was not, suggesting that the nature of membranes of the cells treated with *Bc*-SMase are

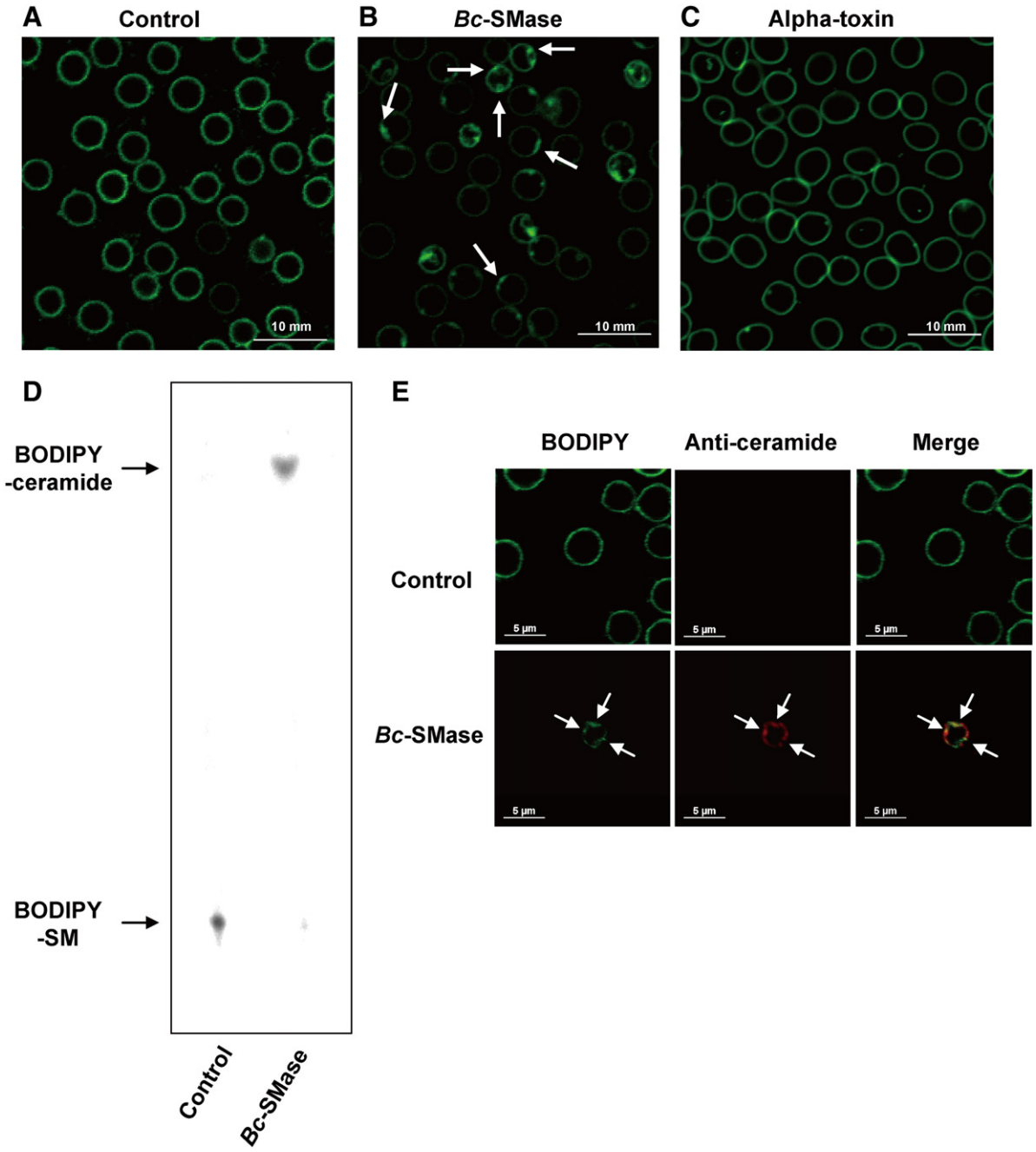

Fig. 6. Confocal fluorescence microscopic analysis of sheep erythrocytes treated with *Bc*-SMase or alpha-toxin. Sheep erythrocytes stained with BODIPY-SM were incubated without (A) or with 0.8 μM *Bc*-SMase (B) or alpha-toxin (C) at 37 °C for 30 min. (D) Identification of BODIPY-labeled sphingolipids in BODIPY-stained erythrocytes treated with or without 0.8 μM *Bc*-SMase. (E) The detection of ceramide-rich domains in BODIPY-stained cells treated with *Bc*-SMase by anti-ceramide antibody. Fluorescences were viewed with a confocal laser microscope. A typical result from one of six experiments is shown.

different from that of untreated cells. These results suggest that fluorescent substance-rich domains in the cells treated with *Bc*-SMase are ceramide-rich domains.

3.8. FRAP measurements of BODIPY diffusion

Klein et al. [32] reported that membrane fluidity of cells was evaluated by measurement of lateral diffusion of fluorescence-labeled SM by FRAP with a confocal laser microscopy. The BODIPY-SM-stained erythrocytes were treated with 0.8 μM *Bc*-SMase, and the rate of effective diffusion of fluorescence substances in ceramide-rich domains or ceramide-poor domains (BODIPY-SM) was measured by FRAP analysis using confocal laser microscopy. Fig. 7B shows a typical sequence of images from a FRAP measurement of BODIPY diffusion. The rate of effective diffusion of fluorescent substances at ceramide-rich domains in erythrocytes treated with *Bc*-SMase decreased to about 50%, compared with that of BODIPY-SM in the untreated cells (Fig. 7B), indicating that the membrane fluidity of the ceramide-rich domains in the erythrocytes treated with *Bc*-SMase did not significantly recover to control level under the experimental condition.

4. Discussion

The present study demonstrated that *Bc*-SMase binds to whole membranes of sheep erythrocytes, and directly hydrolyzes SM in the membranes, and that the formation of ceramide-rich domains by the

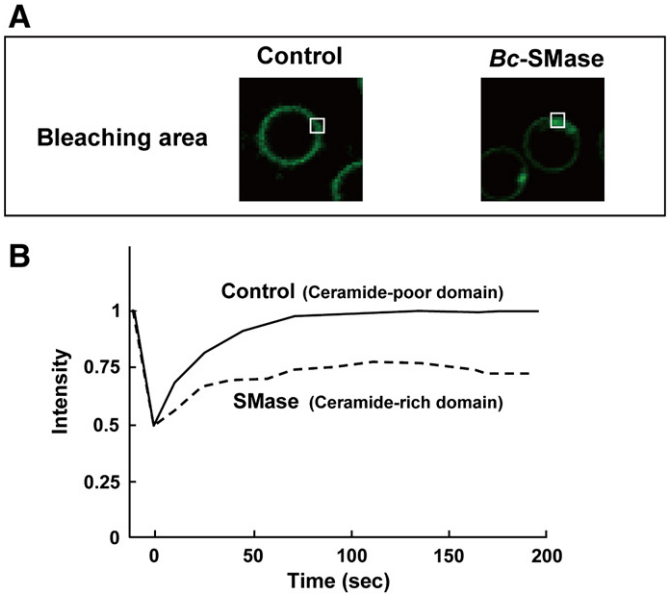

Fig. 7. FRAP analysis of diffusion rate of BODIPY-stained sphingolipids. (A) Bleaching was performed on a 1.0-μm diameter ROI at the indicated region (white square). (B) Representative recovery curves for BODIPY diffusion following control (solid line) or *Bc*-SMase (broken line) treatment are shown. A typical result from one of eighteen experiments is shown.

enzyme leads to a formation of the interface between rigid and fluid domains in the membrane, resulting in their disruption.

We reported that the hemolysis of sheep erythrocytes by alpha-toxin is dependent on the activation of the C24:1-SM metabolic system through activation of endogenous SMase by Gi in DRM, especially the formation of S1P [18,19]. PT did not inhibit the hemolysis or the formation of ceramide from SM induced by *Bc*-SMase in the erythrocytes. The treatment with *N*-OE and DHS had little effect on the hemolysis by *Bc*-SMase. Furthermore, the level of hemolysis by *Bc*-SMase was the same as that by the toxin at concentrations of 0.05–0.8 μM, but the level of ceramide production in membranes of the sheep erythrocytes treated with *Bc*-SMase was about 6- to 15-fold that in erythrocytes treated with alpha-toxin. These results show that the activity of *Bc*-SMase is dependent on the hydrolysis of SM in membranes, but that of the toxin is not. Therefore the mechanism of action of the toxin is linked to the intracellular signal transduction pathway, as reported previously [18,19], while that of *Bc*-SMase is different. When the level of CF-release from liposomes by *Bc*-SMase was the same as that induced by the toxin, the level of ceramide production in liposomes treated with *Bc*-SMase was the same as that in liposomes treated with the toxin, showing that the disruption of artificial membranes, SM-liposomes, induced by the enzyme and the toxin is closely related to production of ceramide from SM. It was found that *Bc*-SMase was highly effective in hydolyzing SM in membranes, compared with the toxin, supporting that the event by *Bc*-SMase is linked to the hydrolysis of SM in membranes.

Alpha-toxin bound almost exclusively to DRM of sheep erythrocytes, and specifically caused the formation of ceramide in DRM [19]. Treatment of the cells with MβCD had in little effect on the hemolysis by *Bc*-SMase, but inhibited that induced by alpha-toxin. *Bc*-SMase bound to not only DRM, but also DSF of sheep erythrocytes. Ceramide formed in the cells treated with *Bc*-SMase was detected in whole membranes, and not inhibited by PT, supporting that *Bc*-SMase hydrolyzes SM in whole membranes. Furthermore, several studies have reported that up to 50% of the total amount of SM in sheep erythrocytes is located in the plasma membrane, being concentrated in the outer leaflet [33,34]. We reported that the β-hairpin region of *Bc*-SMase interacts with phosphorylcholine of SM [15]. Accordingly, it appears that *Bc*-SMase specifically binds to SM in the outer leaflet of membranes of sheep erythrocytes, and hydrolyzes SM to ceramide in membranes.

Treatment of BODIPY-SM-stained erythrocytes with *Bc*-SMase resulted in hydrolysis of BODIPY-SM to BODIPY-ceramide (Fig. 6D), and appearance in fluorescence substance-rich domain (Fig. 6B). Furthermore, the domains, in which fluorescent substance were concentrated, reacted with anti-ceramide antibody (Fig. 6E). These results indicated that fluorescent substance-rich domains in the cells treated with *Bc*-SMase are ceramide-rich domains. FRAP analysis showed that the recovery rate of BODIPY diffusion was reduced in the ceramide-rich domain of sheep erythrocytes treated with *Bc*-SMase, compared with the overall membrane of untreated cells (Fig. 7B), suggesting that formation of the ceramide-rich domains induced by *Bc*-SMase leads to a reduction in the fluidity of the cell membranes. Several studies have reported that the ceramide-rich domain forms a rigid phase in membranes, and that ceramide-induced leakage of aqueous contents from liposomes is due to the structural defects existing at the interface between rigid (ceramide-rich) and fluid (ceramide-poor) domains. [31,35–39]. Therefore, it is possible that hemolysis induced by *Bc*-SMase occurs through the coalescence of ceramide-rich domains and formation of the interface between rigid and fluid domains.

In conclusion, treatment with *Bc*-SMase induces the formation of ceramide-rich domains in membranes, and a decrease in the fluidity of membranes, leading to destabilization under physical stimulation and finally the disruption of membranes.

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